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(21) International Application Number: PCT/US98/22812 (22) International Filing Date: 27 October 1998 (27.10.98) (30) Priority Data: 08/972,563 18 November 1997 (18.11.97) US (71) Applicant (for all designated States except US): BIO-RAD LABORATORIES, INC. [US/US]; P.O Box 5340, Hercules, CA 94547 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WATKINS, Michael, I. [US/US]; 4219 Rolling Hills lane, Vacaville, CA 95688 (US). EDWARDS, Richard, B. [US/US]; 840 Buck Court, Vacaville, CA 95688 (US). (74) Agents: HEINES, M., Henry et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111-3834 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MULTIPLEX FLOW IMMUNOASSAYS WITH MAGNETIC PARTICLES AS SOLID PHASE (57) Abstract <p>Heterogeneous assays for different analytes in a single biological sample are performed simultaneously in a multiplexed assay that combines flow cytometry with the use of magnetic particles as the solid phase and yields an individual result for each analyte. The particles vary in size to encompass distinct size ranges that are distinguishable by flow cytometry, the particles in each size range carrying an assay reagent bonded to the particle surface that is distinct from the assay reagents of particles in other size ranges. The magnetic particles facilitate separation of the solid and liquid phases, permitting the assays to be performed by automated equipment.</p>		

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MULTIPLEX FLOW IMMUNOASSAYS WITH MAGNETIC PARTICLES AS SOLID PHASE

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BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention resides in the field of clinical assays indicative of biological conditions, and is of interest in the technology of binding assays for analytes in biological fluids for purposes of diagnosis, monitoring, or other clinical functions.

10 2. Description of the Prior Art

Since the initial disclosure of radioimmunoassays in 1961, a wide variety of *in vitro* assays using affinity-type binding have been developed. Variations include the type of binding (for example, specific vs. non-specific, and immunological vs. non-immunological), the type of detection (including the use of labels such as enzyme labels, radioactive labels, fluorescent labels, and chemiluminescent labels), methods of detecting
15 whether or not binding has occurred (including methods in which bound species are separated from unbound species and methods that do not include such separation), and various other aspects of the assay procedure. The technology is currently used for the detection and quantitation of countless species, and serves as an analytical tool in the
20 detection and monitoring of many physiological conditions and functions and the diagnosis and treatment of many diseases.

Improvements in the efficiency and reproducibility of these assays have been made by various developments including improved labels, methods of detection, automation, and systems for multiplex analyses. Each procedure however requires a sequence of steps, and
25 any means of shortening the sequence, increasing the number of analyses that can be

performed within a given period of time, or improving the reproducibility and versatility of the assay will benefit the purpose of the assay.

Many binding assays are heterogeneous assays, which rely in part on the transfer of analyte from a liquid sample to a solid phase by the binding of the analyte during the assay to the surface of the solid phase. At some stage of the assay, whose sequence varies depending on the assay protocol, the solid phase and the liquid phase are separated and the determination leading to detection and/or quantitation of the analyte is performed on one of the two separated phases. One type of solid phase that has been used are magnetic particles, which offer the combined advantages of a high surface area and the ability to be temporarily immobilized at the wall of the assay receptacle by imposition of a magnetic field while the liquid phase is aspirated, the solid phase is washed, or both. Descriptions of such particles and their use are found in Forrest *et al.*, United States Patent No. 4,141,687 (Technicon Instruments Corporation, February 27, 1979); Ithakissios, United States Patent No. 4,115,534 (Minnesota Mining and Manufacturing Company, September 19, 1978); Vlieger, A.M., *et al.*, *Analytical Biochemistry* 205:1-7 (1992); Dudley, *Journal of Clinical Immunoassay* 14:77-82 (1991); and Smart, *Journal of Clinical Immunoassay* 15:246-251 (1992).

Of further possible relevance to this invention is the state of the art relating to the use of flow cytometry for the detection and analysis of particles and species bound to the particles. Flow cytometry has been disclosed for use in the detection and separation of antigens and antibodies by Coulter Electronics Inc., United Kingdom Patent No. 1,561,042 (published February 13, 1980); and for quantitation of PCR (Polymerase Chain Reaction) products by Vlieger, A.M., *et al.*, *Analytical Biochemistry* 205:1-7 (1992). Flow cytometry has been limited in the analysis of biological samples. The sensitivity of those assay formats that do not require separation of free from bound species (i.e., sandwich and competitive assays) is adversely affected by the increased background signal noise caused by the unbound label. Antigen-capture antibody assays require the removal of non-specific immunoglobulin before the addition of class-specific labeled anti-Ig. Samples containing particulates (such as stool samples, for example) require the removal of this debris which would otherwise interfere with the flow cytometric measurement. Traditional separation techniques, such as filtration or centrifugation would be successful in removing unbound label or non-specific Ig but would fail to remove interfering particulates from the patient sample. In addition, these traditional separation techniques are difficult and/or costly to automate. The use of magnetic particles and magnetism is a well known method and has been shown to be both efficient and cost-effective in automated diagnostic systems.

SUMMARY OF THE INVENTION

This invention resides in an assay that combines multiplexing of heterogeneous binding assays of a single fluid sample by flow cytometry with the use of solid magnetic particles as the solid phase to facilitate the separation of solid and liquid phases. The magnetic particles have sizes that are microscopic (and hence termed "microparticles") and that vary over a size range that is an aggregate of two or more smaller size ranges, referred to herein as "subranges." The subranges are substantially discrete (nonoverlapping), with the mean particle sizes of adjacent subranges sufficiently far apart to permit differentiation of each subrange from the others by flow cytometry. An assay reagent is bonded to each particle, with substantially all particles within each subrange bearing the same assay reagent and with different assay reagents from one subrange to the next. The subranges are thus distinguishable not only by size for purposes of flow cytometry but also by the assay reagents bonded to the particles such that all particles in each subrange take part in a distinct binding assay, and do so in a selective manner relative to the assay reagents bonded to particles in other subranges.

The invention further resides in a combination of solid particles for use in the multiplex assay described in the preceding paragraph, the particles being of magnetically responsive material that encompass a range of sizes extending over two or more substantially discrete subranges of size that are distinguishable by flow cytometry, the particles bearing assay reagents bonded to their surfaces, with a distinct assay reagent for each subrange.

The magnetic character of the particles permits the automated separation of solid phase from liquid phase at a point in the sequence of the assay prior to the flow cytometry stage. The separation can serve any of a variety of purposes, including the removal of sample debris from the assay components, the removal of sample components that would otherwise contribute significantly to the background noise at the detection stage, the removal of competing binding members that are not the subject of any of the assays but would otherwise interfere with the results, and the removal of bound from unbound species such as labels, analytes, analyte binding members, and label-binding member conjugates. The particular function in any given assay or combination of assays will depend on the nature of the assay and the assay protocol.

These and other features and advantages of the invention will be more readily understood by the description that follows.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The term "magnetically responsive material" is used herein to denote a material that responds to a magnetic field. Magnetically responsive materials of interest in this invention include paramagnetic materials, ferromagnetic materials, ferrimagnetic materials, and metamagnetic materials. Paramagnetic materials are preferred. Examples are iron, nickel, and cobalt, as well as metal oxides such as Fe_3O_4 , $\text{BaFe}_{12}\text{O}_{19}$, CoO , NiO , Mn_2O_3 , Cr_2O_3 , and CoMnP . Rather than constituting the entire microparticle, the magnetically responsive material is preferably only one component of the microparticle whose remainder consists of a polymeric material to which the magnetically responsive material is affixed and which is chemically derivatized to permit attachment of an assay reagent.

The quantity of magnetically responsive material in the microparticle is not critical and can vary over a wide range, although the quantity can affect the density of the microparticle, which in conjunction with the particle size can affect the ease of maintaining the microparticle in suspension for purposes of achieving maximal contact between the liquid and solid phase and for facilitating flow cytometry. Furthermore, an excessive quantity of magnetically responsive material in the microparticles will produce autofluorescence at a level high enough to interfere with the assay results. It is therefore preferred that the concentration of magnetically responsive material be low enough to minimize any autofluorescence emanating from the material. With these considerations in mind, the magnetically responsive material in a microparticle in accordance with this invention preferably ranges from about 1% to about 75% by weight of the particle as a whole. A more preferred weight percent range is from about 2% to about 50%, a still more preferred weight percent range is from about 3% to about 25%, and an even more preferred weight percent range is from about 5% to about 15%. The magnetically responsive material can be dispersed throughout the polymer, applied as a coating on the polymer surface or as one of two or more coatings on the surface, or incorporated or affixed in any other manner that secures the material to the polymer.

The polymeric matrix that forms the remainder of the microparticle can be any material that can be formed into a microparticle, that, other than the assay reagent that is affixed to the microparticle, is inert to the components of the biological sample and to the other assay reagents, that has minimal autofluorescence, that is solid and insoluble in the sample and in any other solvents or carriers used in the assay, and that is capable of affixing an assay reagent to the microparticle. Examples of suitable polymers are polyesters, polyethers, polyolefins, polyalkylene oxides, polyamides, polyurethanes, polysaccharides, celluloses, and polyisoprenes. Crosslinking is useful in many polymers for imparting structural integrity and rigidity to the microparticle.

Functional groups for attachment of the assay reagent can be incorporated into the polymer structure by conventional means, including the use of monomers that contain the functional groups, either as the sole monomer or as a co-monomer. Examples of suitable functional groups are amine groups (—NH_2), ammonium groups (—NH_3^+ or —NR_3^+), hydroxyl groups (—OH), carboxylic acid groups (—COOH), and isocyanate groups (—NCO). A useful monomer for introducing carboxylic acid groups into polyolefins, for example, is acrylic acid or methacrylic acid.

Attachment of the assay reagent to the microparticle surface can be achieved by electrostatic attraction, specific affinity interaction, hydrophobic interaction, or covalent bonding. Covalent bonding is preferred. Linking groups can be used as a means of increasing the density of reactive groups on the microparticle surface and decreasing steric hindrance to increase the range and sensitivity of the assay, or as a means of adding specific types of reactive groups to the microparticle surface to broaden the range of types of assay reagents that can be affixed to the microparticle surface. Examples of suitable useful linking groups are polylysine, polyaspartic acid, polyglutamic acid and polyarginine.

In some cases, particles possess high autofluorescence and as such are unsuitable for use in a flow cytometric immunoassay. Particles created by standard emulsion polymerization techniques from a wide variety of starting monomers in general exhibit low autofluorescence. Particles whose surfaces have been specifically modified to increase porosity and therefore surface area (such particles are referred to in the literature as "macroporous" particles) exhibit high autofluorescence. Autofluorescence in such particles increases with increasing size and increasing percentage of divinylbenzene monomer.

With these considerations in mind, the size range of the microparticles can vary and particular size ranges are not critical to the invention. In most cases, the aggregated size range of the microparticles lies within the range of from about 0.3 micrometers to about 100 micrometers in particle diameter, and preferably within the range of from about 0.5 micrometers to about 40 micrometers. The subranges are two or more in number, and in most cases will be from two to twenty, each selectively active in a single assay and inert relative to the other assays simultaneously being performed or detected.

The widths of the subranges and the spacing between mean diameters of adjacent subranges are selected to permit differentiation of the subranges by flow cytometry, and will be readily apparent to those skilled in the use of and in the instrumentation for flow cytometry. In this specification, the term "mean diameter" refers to a number average diameter. In most cases, a preferred subrange width is about $\pm 5\%$ CV or less of the mean diameter, where CV is the coefficient of variation and is defined as the standard deviation of the particle diameter divided by the mean particle diameter times 100 percent. The minimum spacing between mean diameters among the various subranges can vary depending on the microparticle size distribution, the ease of segregating microparticles by

size for purposes of attaching different assay reagents, and the type and sensitivity of the flow cytometry equipment. In most cases, best results will be achieved when the mean diameters of different subranges are spaced apart by at least about 6% of the mean diameter of one of the subranges, preferably at least about 8% of the mean diameter of one of the subranges and most preferably at least about 10% of the mean diameter of one of the subranges. Another preferred subrange width relation is that in which the standard deviation of the particle diameters within each subrange is less than one third of the separation of the mean diameters of adjacent subranges.

The type of assay reagent attached to the microparticle surface for any single subrange of microparticles will vary depending on both the analyte and the type of assay. The assay reagent can be a binding agent with specific affinity for the analyte, or a binding agent with affinity for a narrow range of species that includes the analyte but excludes other analytes whose assays are performed by contact with other microparticle subranges, or any binding species in general that will selectively engage in the assay for a single analyte to the exclusion of the others. Examples of assay reagents are antibodies, antigens or haptens, and other types of proteins with binding specificity such as avidin and biotin.

Another type of assay reagent that can be attached to the microparticle surface for any single subrange of microparticles is the analyte itself. In the analysis, the attached analyte will compete with a narrow range of species in the sample that also includes analyte. Examples of these assay reagents are antibodies, antigens and haptens.

The signal due to a specific analyte may be differentiated, singly or in combination, by the following parameters: the size of the particle (as reflected in the low and high angle scatter measurements), the composition of the particle (reflected by changes in the high angle scatter), or the fluorescence wavelength of the label (in which the different wavelengths are separated by dichroic filters and detected by the fluorescence photomultiplier tubes).

The assay performed at the surfaces of microparticles within a single subrange can be any type of heterogeneous assay that yields a result differentiating a certain analyte from others in the sample.

Competitive assays, for example, can be performed by using magnetically responsive microparticles to which are bound molecules of a binding protein (such as an antibody) specific for the analyte. During the assay, the sample and a quantity of labeled analyte, either simultaneously or sequentially, are mixed with the microparticles. By using a limited number of binding sites on the microparticles, the assay causes competition between the labeled analyte and the analyte in the sample for the available binding sites. After a suitable incubation period, the mixture of liquid and solid is placed under the influence of a magnetic field, causing the microparticles to adhere to the walls of the reaction vessel, and the liquid phase is removed. The microparticles, still adhering to the

vessel wall, are then washed to remove any remaining unbound analyte and label, and resuspended in a carrier liquid for introduction into a flow cytometer where the microparticles are classified by size and the label detected. An example of an analyte that is readily detected in this manner is vitamin B₁₂. A useful particle-bound assay reagent for this analyte is B₁₂ intrinsic factor, and a competing label-bound analyte is B₁₂ covalently linked to phycoerythrin.

Immunometric or sandwich assays, as another example, are performed by using magnetically responsive microparticles to which are bound antibodies to the analyte. In this case, the bound antibodies are present in excess relative to the suspected quantity range of the analyte so that all of the analyte binds. The microparticles are placed in contact with the sample, and simultaneously or sequentially, a second antibody to same analyte is added, again in excess relative to the analyte, the first and second antibodies binding different epitopes on the analyte in a non-interfering manner, and the second antibody being conjugated to a detectable label. After a suitable incubation period, the liquid mixture with microparticles suspended therein is placed under the influence of a magnetic field, causing the microparticles to adhere to the walls of the reaction vessel, and the liquid phase is removed. The microparticles, still adhering to the vessel wall, are then washed to remove excess amounts of the second, labeled antibody that have not become bound to the immobilized analyte, and the microparticles are then resuspended in a carrier liquid for introduction into a flow cytometer where they are sorted by size and the label detected. An example of an analyte that is readily detected in this manner is thyroid stimulating hormone (TSH). The label on the second antibody can again be phycoerythrin.

This invention can also be applied to assays that do not separate bound label from unbound label but nevertheless require separation of the solid from the liquid phase at some point in the assay. Examples are assays for identifying antibodies to infectious diseases. The analyte antibodies bind to particle-bound antigens in the assay, and are followed by labeled binding members that bind to the analyte antibodies that have thus become attached to the solid phase. The antigens originally bonded to the solid phase are done so in a high density so that any label that subsequently attaches to the solid phase is sufficiently concentrated at the solid phase surface to be distinguishable from unbound label in the solution. While this eliminates the need for separating bound from unbound label, it still requires the removal of other species from the sample that would compete with the labeled secondary antibodies that bind to the analyte antibodies which are bound to the particle-bound antigen. The magnetic particles serve this purpose as in the assays described in the preceding paragraphs, and this is performed before the label is added.

A different type of serological assay for antibodies are a further example, performed by using magnetically responsive microparticles to which are bound antibodies to the antibody analyte. The microparticles are placed in contact with the sample. After a

suitable incubation period, the liquid mixture with suspended microparticles is placed under a magnetic field to adhere the microparticles to the reaction vessel walls, and the liquid phase is removed. Labelled antigen is then added to the vessel containing the microparticles, the antigen being the one that the analyte antibodies are directed towards and that is conjugated to a detectable label or is attached through other binding pairs. After a suitable incubation period, this new liquid mixture is introduced into a flow cytometer where the microparticles are classified by size and the label detected. An example of an analyte susceptible to this type of assay is human anti-Rubella IgG. The particle-bound reagent is Rubella antigen and the labeled secondary antibody is anti-human IgG antibody covalently linked to phycoerythrin.

The multiple assays that can be performed on a single fluid sample in accordance with this invention can be all of the same type (*i.e.*, all competitive, all immunometric, all serological, etc.) or a combination of different types. Examples of combinations of assays that can be performed by this method are:

- (1) Assays for thyroid stimulating hormones and either free T_4 or total T_4 ;
- (2) Assays for vitamin B_{12} and folate; and
- (3) ToRCH assays, detecting serum IgG and serum IgM responses to *Toxoplasma gondii*, Rubella virus, Cytomegalovirus, and Herpes Simplex Virus Types 1 and 2.

Methods of and instrumentation for flow cytometry are known in the art, and those that are known can be used in the practice of the present invention. Flow cytometry in general resides in the passage of a suspension of the microparticles as a stream past electro-optical sensors, in such a manner that only one particle at a time passes the sensors. As each particle passes the sensors, the particle produces a signal due to light scattering, the amplitude of the signal varying with the particle size. The signals are classified by the instrumentation according to their amplitudes, the particles thereby differentiated according to size. The presence and amount of label on each particle is also detected by its fluorescence and correlated with the particle size subrange so that individual assay results are achieved for each particle size subrange. Descriptions of instrumentation and methods for flow cytometry are found in the literature. Examples are McHugh, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," *Methods in Cell Biology* 42, Part B (Academic Press, 1994); McHugh *et al.*, "Microsphere-Based Fluorescence Immunoassays using Flow Cytometry Instrumentation," *Clinical Flow Cytometry*, Bauer, K.D., *et al.*, eds. (Baltimore, Maryland, USA: Williams and Williams, 1993), pp. 535-544; Lindmo *et al.*, "Immunometric Assay Using Mixtures of Two Particle Types of Different Affinity," *J. Immunol. Meth.* 126: 183-189 (1990); McHugh, "Flow Cytometry and the Application of

Microsphere-Based Fluorescence Immunoassays," *Immunochemica* 5: 116 (1991); Horan *et al.*, "Fluid Phase Particle Fluorescence Analysis: Rheumatoid Factor Specificity Evaluated by Laser Flow Cytophotometry," *Immunoassays in the Clinical Laboratory*, 185-189 (Liss 1979); Wilson *et al.*, "A New Microsphere-Based Immunofluorescence Assay Using Flow Cytometry," *J. Immunol. Meth.* 107: 225-230 (1988); Fulwyler *et al.*, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," *Meth. Cell Biol.* 33: 613-629 (1990); Coulter Electronics Inc., United Kingdom Patent No. 1,561,042 (published February 13, 1980); and Steinkamp *et al.*, *Review of Scientific Instruments* 44(9): 1301-1310 (1973).

Similarly, methods of and instrumentation for applying and removing a magnetic field as part of an automated assay are known to those skilled in the art and reported in the literature. Examples of literature reports are the Forrest *et al.* patent, the Ithakissios patent, the Vlieger *et al.* paper, the Dudley paper and the Smart paper, all referenced above in the Description of the Prior Art. All of the citations in this and the preceding paragraph are incorporated herein by reference.

This invention is applicable to the analysis of biological fluids, notably physiological fluids such as whole blood, serum, urine, spinal fluid, saliva, and stool samples.

The following examples are offered strictly for purposes of illustration.

EXAMPLE 1

This example illustrates the attachment of viral antigen (rubella (RUB), cytomegalovirus (CMV) and herpes simplex virus 2 (HSV2)) to magnetic beads.

Three types of magnetic beads were used:

SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/acrylic acid particles), 4.35 micrometers (μm) in diameter, density 1.17 g/cc, containing 12% magnetite (by weight)

SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/acrylic acid particles), 3.18 μm in diameter, density 1.17 g/cc, containing 12% magnetite (by weight)

SINTEF Applied Chemistry, Trondheim, Norway -- poly(styrene/divinylbenzene) particles, 10 μm in diameter, density 1.23 g/cc, containing 17.9% magnetite/maghemite (by weight)

Table I lists the amounts of each of the materials used in this preparation:

TABLE I
Amounts Used

Bead	Viral Antigen	Amount of Beads	Wt. of Viral Antigen	Volume of Viral Antigen	Volume of Phosphate Buffer (100 mM)
4.35 μ m	CMV	10 mg	225.8 μ g	322.6 μ L	677.4 μ L
3.18 μ m	HSV2	5 mg	163.0 μ g	815.0 μ L	185.0 μ L
10 μ m	RUB	5 mg	5.2 μ g	104.0 μ L	896.0 μ L

The beads in each case were placed in test tubes and washed multiple times with 100 mM phosphate buffer, pH 6.8. The washed beads were then suspended in the volume of phosphate buffer listed in Table I, and respective antigen solution was added (CMV antigen from Chemicon International Incorporated, Temecula, California, USA; HSV2 antigen from Ross Southern Labs, Salt Lake City, Utah, USA; and RUB antigen from Viral Antigens, Memphis, Tennessee, USA) in the amount listed in the Table. The test tubes were then rotated in end-over-end fashion overnight at room temperature. The tubes were then placed on a magnetic separator and the supernatant was drawn off and discarded. The resulting beads were washed with a wash buffer consisting of 50 mM phosphate buffer, pH 7.4, 0.01% Tween 20, 1% bovine serum albumin, 0.1% sodium azide, 150 mM sodium chloride, then again subjected to magnetic separation, and suspended in a storage buffer consisting of 50 mM phosphate buffer, pH 7.4, 5% glycerol, 1% bovine serum albumin, 0.1% sodium azide, 150 mM sodium chloride.

EXAMPLE 2

This example illustrates the use of the CMV-coated magnetic beads of Example 1 in a flow cytometric immunoassay.

Procedure:

1. 100 μ L of Bio-Rad CMV IgG Immunoassay positive and negative controls (Bio-Rad Laboratories, Inc., Hercules, California, USA, diluted 1:10 in wash buffer) were added to 12 \times 75 mm polypropylene test tubes.
2. To each tube was added 100 μ L of the CMV antigen-coated particles (described in Example 1) diluted 1:1000 in wash buffer.
3. The tubes were vortexed at ambient temperature for 30 minutes.

4. After vortexing, 800 μ L of wash buffer was added to each tube.
5. The tubes were placed in a magnetic separator for 3 minutes and the liquid phase removed.
6. Steps 4 and 5 are repeated but with 1000 μ L of wash buffer.
- 5 7. 200 μ L of a 1:100 dilution of anti human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) is added.
8. The tubes were vortexed at ambient temperature for 30 minutes.
9. After this time, the samples are injected into the flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a Xenon arc
10 lamp.

Results:

Positive and negative CMV controls exhibited fluorescent peaks corresponding to 898 and 60 relative linear fluorescence units, respectively. As expected, the positive control gave significantly elevated signal relative to that of the negative control.

15

EXAMPLE 3

This example illustrates the use of the CMV, HSV2 and RUB-coated magnetic particles of Example 1 in a simultaneous multi-analyte flow cytometric immunoassay.

Procedure:

1. 100 μ L of patient samples (diluted 1:10 in wash buffer), of known CMV, HSV2
20 and RUB antibody status, were added to 12 \times 75 mm polypropylene test tubes.
2. To each tube was added 100 μ L of a mixture of CMV, HSV2 and RUB antigen-coated particles (described in Example 1) diluted in wash buffer.
3. The tubes were vortexed at ambient temperature for 15 minutes.
4. After vortexing, 800 μ L of wash buffer was added to each tube.
- 25 5. The tubes were placed in a magnetic separator for 5 minutes and the liquid phase removed.
6. Steps 4 and 5 are repeated but with 1000 μ L of wash buffer.
7. 200 μ L of a 1:300 dilution of anti-human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) is added.
- 30 8. The tubes were vortexed at ambient temperature for 15 minutes.

9. After this time, the samples are injected into the flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a Xenon arc lamp.

Results:

- 5 The results are summarized in Table II below:

TABLE II
Test Results

Sample	Antibody Status			Relative Linear Fluorescence Units		
	CMV	HSV2	RUB	CMV	HSV2	RUB
CN6	+	-	+	14	7	155
Cn8	+	-	+	16	6	181
CN12	-	-	+	5	7	240
CN15	-	-	+	5	6	329
23	-	+	-	5	45	43

- 10 The data in Table II show that positive samples have substantially increased fluorescence
15 relative to the negative samples.

EXAMPLE 4

This example illustrates the covalent attachment of rubella (RUB) antigen to magnetic beads.

- 20 The magnetic particles were SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/alkylenic acid particles), 25 mg/mL, 7.1 micrometers in diameter, density 1.165 g/cc, containing 12% magnetite (by weight).

- 25 1.13 mL of beads were placed in a test tube and washed multiple times with 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5. The washed beads were then suspended in 1.25 mL of 2 mg/mL polylysine (MW 18,000) in 50 mM MES buffer, pH 5.5. To the resulting solution was added 125 μ L of 20 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in water. The solution was placed on an end-over-end rotator at ambient temperature for 18 hours. After this time the solution was separated from the particles and discarded. The particles were then washed multiple times with

0.2M borate buffer, pH 8.5. The particles were resuspended in 2.5 mL of borate buffer, pH 8.5. To this solution was added 50 mg of succinic anhydride. The solution was then placed on an end-over-end rotator at ambient temperature for 4 hours. After this time the solution was separated from the particles and discarded. The particles were then washed multiple times with 50 mM MES, pH 5.5. The particles were washed twice with 0.1M carbonate buffer, pH 9.6 and then 3 times with 20mM phosphate buffer, pH 4.5. The particles were finally suspended in 1 mL of 20 mM phosphate buffer, pH 4.5. To this solution was added 1 mL of 20 mg/mL EDC in 20 mM phosphate buffer, pH 4.5. The solution was then placed on an end-over-end rotator at ambient temperature for 4 hours. After this time the solution was separated from the particles and discarded. The particles were washed 3 times with 20mM phosphate buffer, pH 4.5. Afterwards the beads were suspended in 2 mL of 0.2M borate buffer, pH 8.5. To this was added 0.5 mL of 0.2 mg/mL of rubella antigen from Viral Antigens Incorporated, Memphis, Tennessee, USA in 0.2M borate buffer, pH 8.5, 2 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 0.1% sodium azide. The test tube were then rotated in end-over-end fashion overnight at room temperature. The next day, 100 μ L of 0.25M hydroxylamine in 0.2M borate buffer, pH 8.5 was introduced. The solution was then placed on an end-over-end rotator at ambient temperature for 1 hour. After this time the solution was separated from the particles and discarded. The particles were washed 3 times with wash buffer (see Example 1). The resulting beads were taken up in 2.5 mL of wash buffer and placed on an end-over-end rotator at ambient temperature for 1 hour. After this time the solution was separated from the particles and discarded. The particles were washed 3 times with storage buffer (see Example 1). Finally the particles were suspended in 1 mL of storage buffer and placed at 4°C.

25

EXAMPLE 5

This example illustrates the use of magnetic particles with covalently attached rubella antigen of Example 4 in a quantitative flow cytometric immunoassay.

Procedure:

1. 100 μ L of Bio-Rad RUB IgG Immunoassay standards, high positive, low positive and negative controls (Bio-Rad Laboratories, Inc., Hercules, CA, diluted 1:30 in wash buffer), were added to 12x75mm polypropylene test tubes.
2. To each tube was added 100 μ L of the RUB antigen-coated particles (described above) diluted 1:200 in wash buffer.
3. The tubes were vortexed at ambient temperature for 15 minutes.

4. After vortexing, 750 μ L of wash buffer was added to each tube.
5. The tubes were placed in a magnetic separator for 1 minute and the liquid phase removed.
6. Steps 4 and 5 are repeated two more times but with 1000 μ L of wash buffer.
- 5 7. 200 μ L of a 1:300 dilution of anti human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) is added.
8. The tubes were vortexed at ambient temperature for 15 minutes.
9. The samples are then injected into the flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a Xenon/Mercury arc
10 lamp.

Results:

Table III contains data generated by following the above protocol. The standards were fitted to a 4-parameter logistic equation. The concentrations of all samples were calculated from this curve. The values for the controls are similar to the values assigned
15 by the Bio-Rad ELISA technique.

TABLE III
Test Results

Sample	Relative Linear Fluorescence Units	Observed [RUB] (IU/mL)	Reported [RUB] (IU/mL)
Standard 0	21	0	0
20 Standard 1	101	6	8
Standard 2	225	30	30
Standard 3	464	115	96
Standard 4	652	216	240
Standard 5	1171	623	614
25 High Positive	580	174	135
Low Positive	175	18	14
Negative	33	0.3	0.5

The foregoing is offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the operating conditions, materials, procedural steps
30 and other parameters described herein may be further modified or substituted in various ways without departing from the spirit and scope of the invention.

WE CLAIM:

1 1. A method for individually detecting a plurality of analytes in a single fluid
2 biological sample by assays that include the binding of species in said biological sample to
3 a solid phase that is in contact with a liquid medium in which said solid phase is insoluble
4 and the separation of said solid phase from said liquid medium, said method comprising:
5 using as said solid phase a plurality of microparticles of magnetically
6 responsive material each with an assay reagent coupled thereto that is selectively
7 active in an assay for one of said analytes, said microparticles varying in size over
8 a range that is an aggregate of a plurality of subranges, each subrange
9 distinguishable from other subranges of said aggregate by flow cytometry and by
10 the assay reagent coupled to the microparticles of said subrange;
11 magnetically separating microparticles in all of said subranges from said
12 liquid medium; and
13 defining said liquid medium as a first liquid medium, suspending said
14 microparticles separated therefrom in a second liquid medium, analyzing said
15 microparticles in said second liquid medium by flow cytometry in accordance with
16 said plurality of assays, thereby achieving individual detection of said analytes in
17 said biological sample.

1 2. A method in accordance with claim 1 in which one of said assay reagents
2 coupled to said solid phase is a binding protein specific for one of said analytes, said
3 method includes adding to said first liquid medium a detectable label that binds to said
4 solid phase, and said magnetic separation comprises separating detectable label that is
5 bound to said solid phase from unbound detectable label that is suspended in said first
6 liquid medium.

1 3. A method in accordance with claim 2 in which said detectable label is
2 added to said first liquid medium as a conjugate with an additional quantity of said one
3 analyte, causing said conjugate and said one analyte in said sample to compete for said
4 binding protein in a competitive assay.

1 4. A method in accordance with claim 2 in which said detectable label is
2 added to said first liquid medium as a conjugate with an additional quantity of said one
3 analyte after a first incubation period, causing said conjugate to react with those sites of
4 said binding protein not occupied by said one analyte in said sample in a sequential assay.

1 5. A method in accordance with claim 2 in which said binding protein is
2 defined as a first binding protein, and said detectable label is added to said first liquid
3 medium as a conjugate with a second binding protein that is also specific for said one
4 analyte, causing said one analyte to bind to both said first binding protein and said
5 conjugate in a sandwich assay.

1 6. A method in accordance with claim 5 in which said one analyte is an
2 antigen, said first binding protein is a first antibody to said antigen, and said second
3 binding protein is a second antibody to said antigen.

1 7. A method in accordance with claim 5 in which said one analyte is a first
2 antibody, said first binding protein is an antigen, and said second binding protein is a
3 second antibody having binding affinity for said first antibody.

1 8. A method in accordance with claim 1 in which one of said assay reagents
2 is a binding protein bound to said solid phase and specific for one of said analytes and for
3 other components in said first liquid medium, and said magnetic separation comprises
4 separating said microparticles with said one analyte bound thereto from said other
5 components in said first liquid medium prior to contacting said microparticles with a
6 second liquid medium containing a detectable label that binds specifically to said one
7 analyte.

1 9. A composition comprising a plurality of solid-phase assay reagents
2 selectively active in a plurality of assays each for a different analyte, each said solid-phase
3 assay reagent comprising a binding species that is selective active in a single assay and
4 coupled to one of a plurality of microparticles of magnetically responsive material, the
5 sizes of said microparticles varying in size over a range that is an aggregate of a plurality
6 of subranges, each subrange distinguishable from other subranges of said aggregate by
7 flow cytometry and by the binding species coupled thereto.

1 10. A composition in accordance with claim 9 in which said microparticles are
2 comprised of a combination of a polymer and a paramagnetic substance, said polymer
3 being formed from monomers including carboxylate groups to permit covalent bonding of
4 assay binding members at the microparticle surface.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22812

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N15/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 1 561 042 A (COULTER ELECTRONICS INC.) 13 February 1980 cited in the application see the whole document --- -/--	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/22812

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DE 44 27 821 A (SILICA GEL GMBH ADSORPTIONS-TECHNIK) 1 February 1996 see the whole document -----	1-10

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Information on patent family members

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